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APPLICATION NUMBER: ***60/555,815***
FILING DATE: ***March 24, 2004***

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EL 717 745 489 US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Marisa E.E.	Jaconi	Geneva, Switzerland

Additional inventors are being named on the one *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

3D-CARDIAC TISSUE ENGINEERING FOR THE CELL THERAPY OF HEART FAILURE

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification *Number of Pages* 22
☑ Drawing(s) *Number of Sheets* 1
☑ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☑ Other (specify) Return Postcard

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Respectfully submitted,

SIGNATURE Rivka D. Monheit
Date March 24, 2004
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REGISTRATION NO. 48,731 *(if appropriate)*
Docket Number: KUROS 110
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Docket Number	KUROS 110

INVENTOR(S)/APPLICANT(S)

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Prisca	Zammaretti-Schär	Zürich, Switzerland

Number 1 of 1

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FEE TRANSMITTAL for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

☑ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 80.00

Complete if Known	
Application Number	
Filing Date	March 24, 2004
First Named Inventor	Marisa E.E. Jaconi
Examiner Name	
Art Unit	
Attorney Docket No.	KUROS 110

METHOD OF PAYMENT *(check all that apply)*

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☑ Deposit Account:

Deposit Account Number: 50-1868

Deposit Account Name: Holland & Knight LLP

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FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
1004	770	2004	385	Reissue filing fee	
1005	160	2005	80	Provisional filing fee	80.00

SUBTOTAL (1) ($) 80.00

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

	Extra Claims		Fee from below	Fee Paid
Total Claims	-	-20* =		X =
Independent Claims		-3** =		X =
Multiple Dependent				=

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
1202	18	2202	9	Claims in excess of 20
1201	86	2201	43	Independent claims in excess of 3
1203	290	2203	145	Multiple dependent claim, if not paid
1204	86	2204	43	** Reissue independent claims over original patent
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SUBTOTAL (2) ($) 0.00

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FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for *ex parte* reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
1502	480	2502	240	Design issue fee	
1503	640	2503	320	Plant issue fee	
1460	130	1460	130	Petitions to the Commissioner	
1807	50	1807	50	Processing fee under 37 CFR 1.17(q)	
1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
1809	770	2809	385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
1801	770	2801	385	Request for Continued Examination (RCE)	
1802	900	1802	900	Request for expedited examination of a design application	

Other fee (specify) _____

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SUBMITTED BY

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We claim:

1. A composition for repair of cardiac tissue comprising a biodegradable matrix, one or more active agents, and stem cells differentiating to form cardiac tissue.

2. The composition of claim 1 wherein the matrix is formed of a gel.

3. The composition of claim 2, wherein the biodegradable matrix is formed of a material selected from the group consisting of fibrin, collagen, and synthetic polymers mimicking the extra cellular matrix (ECM).

4. The composition of claim 1, wherein the stem cells are embryonic, fetal or adult stem cells.

5. The composition of claim 1, wherein the active agents are selected from the group consisting of growth factors and cytokines.

6. A cardiac patch comprising a fibrin matrix, one or more active agents, and stem cells.

7. A method for repair of heart tissue comprising implanting into the heart tissue any of the compositions of claims 1-6,.

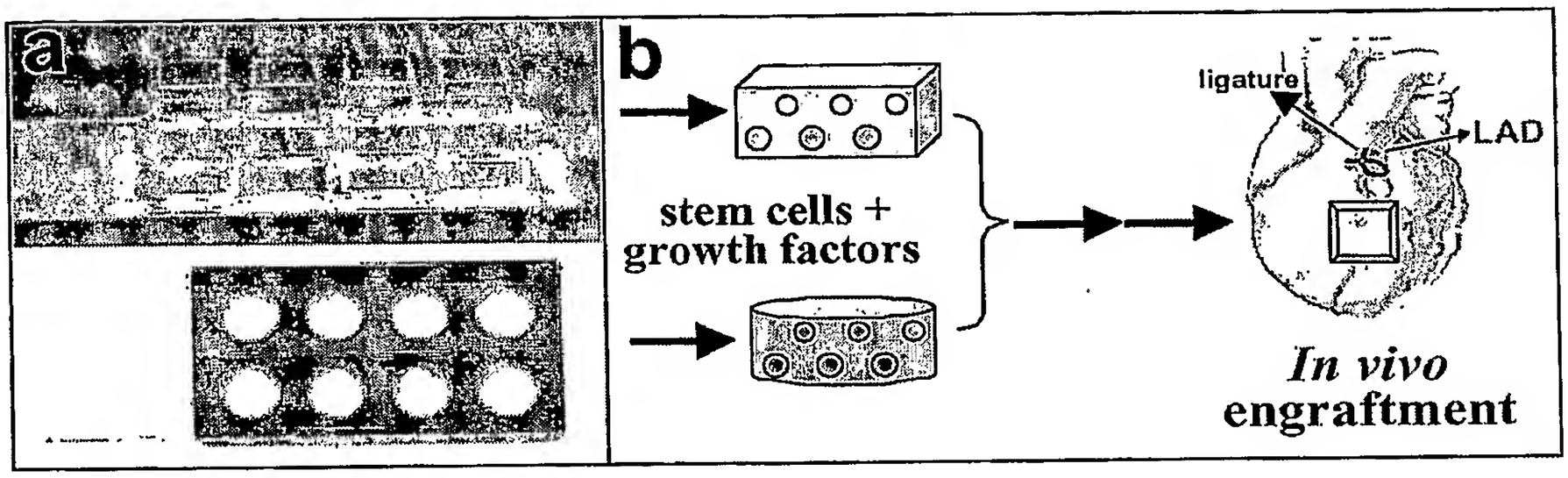

Figure 1. Technique for the *in vitro* 3D-tissue engineered fibrin gels.
(a) Different multi-well systems are used to produced 3D fibrin gels containing appropriate types of stem cells and growth factors to study their differentiation and tissue formation *in vitro*. **(b)** Such engineered "cardiac patches" are the implanted onto normal or infarcted rat hearts.

APPLICATION DATA SHEET

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Application Information:

Title Line One::	3D-CARDIAC TISSUE ENGINEERING FOR THE
Title Line Two::	CELL THERAPY OF HEART FAILURE
Title Line Three::	
Total Drawing Sheets::	1
Formal Drawings?::	No
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Licensed US Govt. Agency::	
Contract or Grant Numbers One::	
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Secrecy Order in Parent Appl.?::	No

Representative Information

Representative Customer Number::	23579

Title: *"3D-Cardiac Tissue Engineering For The Cell Therapy Of Heart Failure"*
By: Marisa E.E. Jaconi and Prisca Zammaretti-Schär
Filed: March 24, 2004

CERTIFICATE OF MAILING UNDER 37 CFR §1.10

I hereby certify that this **PROVISIONAL APPLICATION**, and any documents referred to as attached therein, are being deposited with the United States Postal Service on this date, March 24, 2004, in an envelope as "Express Mail Post Office to Addressee" service under 37 CFR §1.10, Mailing Label Number EL 717 745 489 US, addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450.

Pam Turnbough

Date: March 24, 2004

1804373_v1

PROVISIONAL PATENT APPLICATION

BY

MARISA E.E. JACONI

AND

PRISCA ZAMMARETTI-SCHÄR

FOR

3D-CARDIAC TISSUE ENGINEERING FOR THE CELL THERAPY OF HEART FAILURE

1803072_v1

3D-CARDIAC TISSUE ENGINEERING FOR THE CELL THERAPY OF HEART FAILURE

Background of the Invention

The invention is generally in the field of tissue engineering and in particular relates to methods and compositions for repair of heart tissue by implantation of stem cell.

New steps in regenerative medicine are showing the first advances in the application of stem cells for tissue regeneration. Up to now, cardiac tissue engineering utilizes cells seeded into polymeric scaffolds to try to reproduce the myocardial structure and properties. However, among other problems, neovascularization is still a limiting factor while using conventional tissue scaffolds. Heart failure caused by myocardial infarction may be successfully treated in human beings by cell therapy using a multidisciplinary approach combining expertise in stem cells biology, tissue engineering as well as non-invasive cardiac imaging.

Over the past few years, research on animal and human stem cells (either embryonic, fetal or adult stem cells) has experienced tremendous advances which are almost daily loudly revealed to the public on the front-page of newspapers. The reason for such an enthusiasm over stem cells is that they could be used to cure patients suffering from spontaneous or injuries-related diseases that are due to particular types of cells functioning incorrectly, such as cardiomyopathy, diabetes mellitus, osteoporosis, cancers, Parkinson's disease, spinal cord injuries or genetic abnormalities. This new concept of "regenerative medicine" by stem cells therapies is unprecedented since it involves the regeneration of normal cells, tissues and organs which could allow treating patients.

Embryonic stem cells derived form the embryo at the blastocyst stage are defined as pluripotent since they have the tremendous ability to self-renew and also to differentiate into a variety of cells of all 3 germ layers [1]. Adult stem cells present in some organs of the adult body also have some regenerative capacities (i.e. satellite cells and bone marrow cells). However their potential to

transdifferentiate to phenotypes different from those to which they are pre-committed seems so far more restricted [2,3]. For this reason, they are defined as multipotent. On the other hand, these cells would allow autologous cell transplantation avoiding problems of rejection and immunosuppressive drugs. Therefore, research on both type of stem cells is complementary and needs to be pursued in parallel.

Heart failure is the number one cause of death in industrialized countries. Myocardial infarction typically results in fibrotic scar formation and permanently impaired cardiac function because, after a massive cell loss due to ischemia, the myocardial tissue lacks intrinsic regenerative capability. Thus, efforts to regenerate functional myocardial tissue are being firstly pursued through cell grafting. The principal feasibility of cardiac cell implantation in the heart has been achieved nearly 10 years ago [4-6]. Thereafter, several groups have enlarged our knowledge about the fate of implanted cells of various origin (embryonic, fetal or adult) in the myocardium of healthy and diseased heart (reviewed in [7]. Most studies support the notion that cell engraftment in models of myocardial infarction can improve contractile function [8]. There are presently several ongoing clinical studies in humans using adult stem cells (skeletal myoblasts, bone marrow stem cells) to investigate the safety and feasibility of such a cardiac cell therapy [9]. However, there is still no convincing demonstration for a transdifferentiation to the cardiac phenotype of such adult stem cells. So far, first clinical results are still controversial and demonstrated the need to better understand the stem cell biology.

Cardiac tissue engineering includes the fields of material sciences and cell biology. It has emerged as an alternative promising approach to replace or support an infarcted cardiac tissue and thus may hold a great potential to treat and save the lives of patients with heart diseases. Tissue engineering involves the construction of tissue equivalents from donor cells seeded within 3D biomaterials, then culturing and implanting the cell-seeded scaffolds to induce and direct the growth of new, healthy tissue. Tissue-engineering technologies

have therefore the potential to revolutionize soft-tissue reconstruction by creating biologically based tissue replacements.

After myocardial infarction, the lost cardiomyocytes due to a lack of vascularization are replaced by a scare formed by fibrotic tissue. The surviving cardiac cells undergo neurohormonal-induced hypertrophy as a compensatory mechanism in order to maintain a demanded cardiac output. The heart wall becomes thinner and the ventricle dilates, leading to an end-stage congestive heart failure. Therefore the possibility to repopulate these infarcted areas would be essential for the restoration of a functional contracting myocardium.

Repopulation of these tissues by the application of extracellular matrix-based gels engineered with stem cells is the goal. The plasticity of the stem cells and the presence of appropriate cytokines and growth factors will enable to guide the differentiation of the stem cells into functional cardiomyocytes. Furthermore, the addition of other growth factors will enable us to provide the region with an enhanced vascularization, necessary for the viability of the patch and the integration into the myocardium.

Cardiac tissue engineering methods typically utilize cells seeded into or onto polymeric scaffolds [10-12]. Reproducing the special organizational, mechanical, and elastic properties of native myocardium represents a significant challenge from the perspective of tissue engineering scaffolds. Ideally, these constructs display properties of native myocardium such as coherent contractions, low diastolic tension, and syncytial propagation of action potentials. To be applicable for surgical repair of diseased myocardium engineered tissue constructs should have the propensity to integrate and remain contractile *in vivo*. In order to better meet the mechanical demands of force-generating contractile tissue, biodegradable materials such as hydrogels may serve as more appropriate scaffolds for transplantation of organized cardiac tissue constructs to the heart for myocardial repair.

Recent attempts to engineer soft tissue include the use of scaffolds manufactured from natural polymers, such as collagen,[13] gelatin, alginate [19] hyaluronic acid [20], and chitosan [21] gels or synthetic, biodegradable polymers [22,23].

So far, various methods to produce 3D-cardiac tissue constructs have been proposed [13-18], but the reconstruction of a functional heart tissue remains to be achieved. In particular, problems with vascularization still limit the use of conventional tissue scaffolds in the replacement of large-size tissue defects.

Clearly, cardiac tissue engineering for replacement therapy has an emerging technology still in its early days due to the complexity of the heart tissue. Its true value remains to be thoroughly evaluated.

It is therefore an object of the present invention to create 3D-engineering gels allowing angiogenic and cardiac differentiation of trapped stem cells for the therapy of heart diseases.

Summary of the Invention

Cardiac patches have been developed using extracellular-based gels containing stem cells positioned on a heart surface as a cell therapy to treat heart failure and renewal of infarcted tissue. The incorporation of bioactive and biodegradable materials locally releasing appropriate growth factors and cytokines into the material in which stem cells are seeded support and improve the differentiation of embryonic stem cells (ESC) into viable cardiomyocytes and allow endothelial progenitor cells (EPCs) to build a vascular bed.

Detailed Description of the Invention

A. Gel-Based Matrices and Biomaterials

Bioactive materials are materials which incorporate growth factors or cytokines and are able to release them either at a controlled ratio or directly on cell demand. Extracellular matrix (ECM) components like fibrin or collagen, or synthetic polymers mimicking the ECM, like the polyethylenglycol-modified polymers, can be used as vehicles for cell transplantation. These materials offer the cells an environment providing adhesion natural sites, eventually growth factors and cleavage sites enabling the cells to substitute the material while proliferating and migrating.

Native ECMs components such as fibrinogen from human plasma are particularly useful since it can be cleaved by thrombin into its fibrin subunits. After cleavage a self-assembly step occurs in which the fibrinogen monomer

come together and form a non-covalently cross-linked gel via proteolytic exposure of binding sites. The covalent crosslinking of the chains is achieved via factor XIIIa, which is a trasglutaminase. Through factor XIIIa it was shown that other proteins like fibronectin or collagen can be bound [24-26].

By protein-engineering it is possible to produce growth factors and cytokines, which has an alpha2-plasmin inhibitor sequence in their N-terminus. Gels containing such "architectures, can trap growth factors and cytokines, which can be subsequently cleaved on cell demand [27,28]. Therefore, the degradation ratio of the gel as well as the release of growth factors will be cell proliferation dependent.

B. Cells for cardiac repair

Cardiomyocytes are the main cellular component but non-muscle cells (such as endothelial cells, fibroblasts, smooth muscle cells and leukocytes) play an important role in cardiac development and function [29-31]. The exact contribution of each single cell type to tissue-formation has not been deeply analyzed yet, but the data strongly suggest that formation of a true cardiac tissue-like 3D construct requires the presence of cardiac myocytes and non-myocytes in physiological mix.

1) <u>Cardiomyocytes derived from ESC</u>

Embryonic stem cells (ESC) are pluripotent cells derived from the inner cell mass of the blastocyst. Therefore they have the ability to differentiate into many kind of cell lineage, a capacity that becomes progressively restricted with development. Unlimited differentiation capacity and indefinite propagation represent the strongest advantages of ESC.

Pluripotent ESC are able to differentiate *in vitro* into structures called embryoid bodies (EBs). During this differentiation process, ESCs develop into a variety of committed cell lineages originating from mesoderm, ectoderm and endoderm. Therefore, the possibility to isolate, from differentiating, embryonic cardiomyocytes that are still in a proliferative phase [32] offers a tremendous advantage. Several studies have demonstrated the possibility to engraft such cells into animal models of heart failure [4,7] with encouraging results. Since the derivation in 1998 of human ESCs lines from pre-implantation embryos [33], considerable research is centered on their biology, in particular on how to encourage a specific cell differentiation [34,35] and also on means to enrich and purify derivative cell types such as cardiomyocytes [36]. Autograft cells are preferred, but allograft cells can be utilized, with appropriate selection and utilization of immunosuppressants, such as those currently in use following tissue or organ transplantation.

2) <u>EPCs-dependent vascularization</u>

Endothelial progenitor cells (EPCs) isolated from bone marrow [37], peripheral [38] or cord blood [39] were shown to play a crucial role in many physiological and pathological situations. These cells participate to complex processes like angiogenesis and arteriogenesis [40], which are important steps in vascular repair. The involvement of these cells in the neovascularization of tissues was observed in animal models during tumor development [41] as well as during therapeutic angiogenic assays [42]. These cells have shown promising results when transplanted in ischemic limb [43] and into the heart [44].

EPCs are known for their high proliferation rate and capability to support angiogenesis and revascularization in ischemic tissues [37,43] and differentiate into

the terminal phenotype of endothelial cells (ECs). Their proliferative potential, when compared to the one of endothelial cells, as well as their differentiation pathways into endothelial cells, smooth muscle cells and cardiomyocytes, makes them suitable candidates for the implantation in infracted hearts. However, to completely restore cardiac function in infracted areas EPCs seems not to be sufficient, because it was shown that only in presence of cardiomyocytes, this transdifferentiation is possible.

Although many publications in the field of therapeutic angiogenesis appeared during the last years, few were dedicated to the study of the interaction of EPCs, the extracellular matrix (ECM) and the growth factors. The assessment of stem cell therapy requires serial measurements of myocardial function, perfusion, viability and cell tracking.

C. Method of Implanting and Assessing Function

Echocardiography is well established to measure myocardial function. Single photon emission computed tomography (SPECT) and positron emission tomography (PET) can also determine myocardial perfusion and metabolism. However, they are not able to resolve transmural analysis of the myocardium wall. In addition, dedicated set-up is needed for small animal imagings that are not widely available.

Recently, magnetic resonance imaging (MRI) has emerged as a promising and recognized tool for cardiac imaging as it will be further developed. MRI is now accepted as a gold standard for the assessment of the cardiac anatomy and volumes including the ejection fraction (EF) and cardiac output. Cine MRI provides a highly reproducible protocol to assess the myocardial contraction. Using special saturation pulses, the myocardium can be tagged for quantitative measurement of regional strains [46]. MRI is also very sensitive to myocardial perfusion when first-pass contrast media study are performed [47]. Finally, new MRI myocardial viability methods have been validated using contrast media as gadolinium chelates induce a hyperenhancement of infarct myocardium [48]. MRI assessment of viability

correlates with PET for transmural infarct but appears to detect subendocardial infarction missed by PET [49].

As the MRI contrast and resolution between types of cells is limited, a new strategy has been developed to monitor *in vivo* specific groups of cells. Since the *in vivo* uptake of MRI contrast media can not be tailored to any types of cells, *ex-vivo* cells are typically labeled using paramagnetic or superparamagnetic iron oxide (SPIO) contrast media. After implantation, the labeled cells locally destroy the magnetic field homogeneity. This results on dark spots that can be detected using dedicated MRI sequences [50]. However, the assessment is largely qualitative based on the presence or absence of the dark spots. A quantitative assessment of the contrast media and cells distribution in the tissue remains a significant and largely unresolved challenge.

***In vitro* cell labeling**

Presently, two types of MR contrast agents are used clinically: gadolinium-analogues and iron oxide nanoparticles. These agents are particularly designed as blood-pool contrast agents which are impermeable to cells. Recently, superparamagnetic nanoparticles of anionic γ-Fe_2O_3 (iron oxide) have been shown to label efficiently different types of cells due to the negative surfaces charges. These particles are thus particularly suited for cellular imaging *in vivo*, due to a near-cellular (i.e. 20-50 microns) resolution, long half-life and low local or systemic toxicity. However their uptake by cells still needs to be improved, for example using lipofection agents [51,52]. A preferred magnetic labeling approach is based on the use of the FDA-approved SPIO formulation Ferridex, mixed with a transfection agent [51]. A recent work by Hoehn *et al* used such a labeling to track GFP-expressing ESC (green fluorescent protein), by MRI upon implantation into an ischemic rat brain [52].

***In vivo* cell tracing in the heart**

So far, two studies have already demonstrated the feasibility for the *in vivo* detection of labeled stem cells in the heart [53]. Both studies used a swine model of myocardial infarction on a limited number of animals. Injection of 10^6 labeled stem cells generated detectable signal void using clinical 1.5T MRI

scanner that was no longer visible at 3 weeks [53]. The effect of the stem cells on the myocardial infarct was not assessed in these preliminary studies.

The present invention will be further understood with reference to the following studies.

Example 1: Preparation of Cardiac Patches

Methods and preliminary results

Experiments were performed which establish the feasibility of growing mouse ESC, as well as differentiating ESC within embryoid bodies and neonatal cardiomyocytes into 3D-fibrin gels. Ongoing studies on the differentiation of EPC show that the presence of vascular endothelial growth factor (VEGF) is preferred for their differentiation into mature endothelial cells. Indeed, EPC have the capability to create tube-like constructs in fibrin gels in presence of the epidermal growth factor (EGF).

In vivo properties of the 3D cardiac patches were studied. One fundamental question is to investigate the fate of stem cells after implantation to (i) map the spatial distribution, (ii) measure the rate of migration *in situ*, and (iii) monitor the survival of the grafted cardiac patches. To this end, protocols are drawn up for the labeling of cells with nanoparticles of iron oxide in order to study their biology *in vitro* and to trace them after implantation *in vivo*.

1) Set-up of *in vitro* cells labeling protocols using MRI contrast media. First, commercially available contrast media will be used for toxicity and adverse effects on cellular function studies. In a second step, new engineered contrast media are investigated to improve the specificity of the cells labeling.

2) Development of a quantitative MRI protocol to measure the amount of contrast media inside the cells.

3) Assessment of the *in vivo* spatial and temporal distribution of the labeled cells.

These new contrast media derived from iron nanoparticles are coated with specific antibodies or peptides. The engineered surface of the contrast media are thus modified to improve the internalization of the product. Enhanced efficiency as well as increased specificity of the internalization is expected. In

addition, fluorescein probe are also added to obtain a dual contrast agent that enables the live cell monitoring and ease the correlation between histopathological examination and MRI.

Methods and preliminary results:

Prussian blue staining, electron microscopy and spectrophotometry to evaluate the nanoparticles inside the cells and determine the tolerance of the ES cells to the paramagnetic label are used.

The feasibility of rat heart imaging by MRI at 1.5 T are illustrated by the performance of serial *in vitro* MR imaging of labeled cells suspended in fibrin gels within a spectrophotometric cuvette to develop a quantitative protocol to measure the iron content inside the cells. This MR protocol is applied to measure the time evolution of the iron concentration *in vivo* after the cardiac patches engraftment.

The applicability of cell containing-3D fibrin gels into infarcted areas of a rat model of myocardial infarction in order to replace nonfunctional diseased cardiac tissue are evaluated, in particular by performing *in vivo* MRI monitoring of the 3D-labeled cardiac patches after heart implantation to define the bio-distribution of the stem cells in correlation to the cardiac function and infarct evolution.

Examining *ex-vivo* the transplanted heart by immunohistochemical techniques to evaluate cell fate and biodistribution.

Methods and preliminary results:

In preliminary tests the feasibility of fixing empty fibrin gels on the left ventricle of normal hearts was examined. Furthermore, in a first attempt to engraft a gel containing undifferentiated ESC, cell survival and proliferation were observed for up to 2 weeks.

Materials and Methods

The 3-D gel systems used for manufacture of the cardiac patches are based on extracellular matrix proteins, the gel systems have the following properties;

E-Modulus (elastic characteristics): 30-80kPa

Type of material: gel-like material with high water content, typically of 90 to 95%.

The 3D gel systems are designed so that different types of cells can be employed and combined within the gels: (i) embryonic stem cells committed to a cardiac phenotype, and (ii) adult stem cells (such as endothelial progenitors cells) , able to improve the neovascularization of the damaged tissue and/or to transdifferentiate to the cardiac phenotype.

Example of adult stem cells: isolation of MNCs and CD34+ cells from human umbilical cord blood (UCB)

UCB cell collection was approved by the ethical committee of the University Hospital Zurich. Typically, 50 mL of UCB could be collected from fresh placentas with umbilical cord into Vacutainer tubes containing citrate as anticoagulant. The UCB was diluted with two volumes of Ca^{2+} - and Mg^{2+} -free Dulbecco PBS (D-PBS). Mononuclear cells (MNCs) were isolated by density gradient centrifugation with Biocoll (Oxid AG, Basel, Switzerland), then washed 3 x in D-PBS. Positive selection of CD34+ was performed by a magnetic bead separation method (MACS; Miltenyi Biotec, Gladbach, Germany), using the manufacturer's protocol.

Culture of Endothelial Progenitor Cells (EPCs)

2×10^4 MACS-selected CD133+ cells were plated on 8-well glass culture dishes (Nunc Lab-Tek^{TM}II Chamber slide system; VWR International AG, Dietikon, Switzerland) coated with 10 ng/mL human fibronectin (Bioreba, Basel, Switzerland) and 1% gelatin, or fibrin gel substrates. Cells were seeded in endothelial cell growth medium (EC) (C-22010; Clontech, Palo Alto, CA; this medium initially contains 2% FBS and the additive C-39215) supplemented to 20% fetal calf serum (FCS). Cultures were grown at 37°C, 5% CO_2, in a humidified atmosphere. After 24 hr, the non-adherent cells were removed from adherent cells via careful replacement of medium with fresh EC medium.

Growth of EPCs on two-dimensional fibrin gel for cardiac implants

100 µL fibrin gel substrates were formed at the bottom glass of tissue culture chambers (Nunc Lab-Tek^{TM}II Chamber slide system). Epidermal growth

factor (EGF), or $VEGF_{165}$ were admixed to fibrin gel substrate at 150 ng/mL gel. Control fibrin substrates were prepared with neither growth factor. 2×10^4 MACS-selected CD133+ cells were seeded in 400 µL Clontech EC medium atop the fibrin gel substrates formulated with growth factors. Assuming an even distribution of freely diffusing EGF or $VEGF_{165}$ between fibrin gel substrate and overlaying culture medium, this resulted in an initial concentration of 50 ng/mL EGF or $VEGF_{165}$ in the culture system. The culture medium was changed after 24 hr. A second change of medium after another 48 hr was found critical for cell survival. Subsequent changes of EC culture medium were performed after 48 hr or 72 hr. In each change, 350 µL culture supernatant were removed and replaced with an equal volume of fresh EC medium. These changes of medium resulted in removal of EGF and VEGF and the concomitant decrease of EGF or $VEGF_{165}$ concentrations in the culture system. The concentrations of growth factors were calculated to be 21 ng/mL after the first change of medium, and 8.8 ng/mL, 3.7 ng/mL and 1.5 ng/mL after the second, third, and fourth change, respectively. After 14 days of cultivation a substantial part of the gel was proteolized, therefore, in order to implant the gels on the top of the infracted area a 1% fibrin gel was added on top of the cells. After extraction of the gel from the well, these were implanted in the infracted area and fixed with two sutures on the myocardium.

An analogous procedure was applied for embryonic stem cells and other adult stem/progenitor cells used. ESC stable clones containing the CD63-GFP marker gene were used to isolate green ESC-derived cardiomyocytes upon differentiation within embryoid bodies. 3D-fibrin gels containing undifferentiated CD63-GFP ESC were monitored at day 0 and 4 days later. This allowed researchers to follow their fate *in vitro* within the gels (either alone or in combination with EPCs), as well as to identify them *in situ* after their engraftment. Gels containing embryoid bodies (EBs) formed by mouse ESC were preincubated for 6 days to allow cardiac differentiation. With time (up to 6 days) the EBs within the gels spread and cells migrated. Similarly, freshly dissociated and purified neonatal rat cardiomyocytes redifferentiate over time

(up to 14 days) in the 3D gels and remain viable for weeks in culture, forming a network of spontaneously beating cells.

Example 2: Implantation in a Rat model of myocardial infarction

Male Sprague-Dawley rats weighing 300-350 grams were initially anesthetized with 4-5% isofluorane in an induction chamber. Following the shaving and weighting, the rat was intubated with a 14-gauge catheter, tracheal ventilation was performed at 70 cycles/min with 2.5-3.0mL tidal volume, room air supplemented with oxygen (Harvard Rodent Ventilator, Model 683, Harvard Apparatus Co, Inc). 1.5-2% isofluorane was maintained for continuous anesthesia.

Three electrodes were positioned to record the electrocardiographic tracing (ECG) monitor. The respiration curve was also recorded during all procedure.

A left intercostal thoracotomy was performed under aseptic technique. The fourth intercostal space was opened carefully to avoid accidentally cutting any vessels including the internal mammary artery. The fourth and fifth ribs were separated with a small retractor (Harvard Apparatus, France) to explore the heart. The pericardium was removed, the left anterior descending artery and its branch was observed under surgical microscope. A 6-0 polypropylene snare was made passing through the epicardium layer around the origin of the artery between the left atrium and the right pulmonary outflow tract, tying the ligature permanently occluded the artery. After LAD ligature, the left ventrical anterior free wall becomes hypokenetic and clearer due to the cyanosis.

The muscle layer and skin were closed with 3-0 suture afterwards. Before the rat woke up completely, extubation was performed and the rat was places in a recovery cage with a supply of oxygen for 30 to 60 minutes.

Transplantation Procedure

3D cardiac patches implantation was performed into two different conditions of the rats: with or without myocardial infarction. Echo study was performed for left ventricular function evaluation in MI rats. 1 week, 4 or 8 weeks after implantation, the rats were sacrificed and the histologic and

pathologic studies were performed. To evaluate the transformation of left ventricular function, the echocardiograph study was performed to the rats with infarction the day before cells grafting and the day before sacrificing respectively.

Engraftments were performed on the normal hearts, immediately after coronary ligation, 1-week or 4 weeks after myocardial infarction. After 2 weeks, the immunohistochemistry staining revealed a gel full of proliferating ESC, positive for the proliferating cell nuclear antigen (PCNA) and the GFP marker.

Rats were anesthetized, and under sterile technique, the chest was re-opened. The infarcted area was identified visually on the basis of surface scar and wall motion abnormality. 3D patches are applied and fixed onto the of the left ventricular anterior free wall. Control animals received empty 3D patches.

Immunosuppression treatment

To prevent rejection and assess the effect of an immunosuppression treatment on stem cell fate, groups of rats received immunosupression agent cyclosporin A delivered continuously via an osmotic minipump (ALZA Corporation). Alzet mini-osmotic pumps were filled with cyclosporin A (CsA) (Sandimmune, Novartis 50mg/1ml), and was kept overnight at 37°C in PBS before implantation. The CsA release was adjusted at 2.5µl/hour or 10µl/hour and pump were designed for a 7- or 28-days release. The administrated dosage of CsA was calculated as 6-9 mg/Kg/day. After hair shaving and skin cleaning at the site for incision, a hemostat was inserted into the incision to spread the subcutaneous tissue and create a prompt pocket for the pump. The filled pump was implanted subcutaneously and the wound closed with suture. All procedure was performed under sterile circumstances.

Evaluation of the left ventricular function by echocardiography

For the evaluation of left ventricular function, transthoracic echocardiogram was performed on the rats after myocardial infarction 1 week or 4 weeks right before implantation (baseline echocardiogram), and 1 week or 4 weeks after implantation, before the sacrifice of the animals. Rats were

anesthetized with 4-5% isofluorane in an induction chamber. The chest was shaved, the rats were placed in dorsal decubitus position and intubated for continuous ventilation. 1-2% isofluorane was continuously supplied via a mask. 3 electrodes were adhered to their paws to record the electrocardiographic tracing simultaneously with the cardiac image identifying the phase of a cardiac cycle.

Echocardiograms were performed with a commercially available echocardiography system equipped with 7.5 MHz phased–array transducer (Philips-Hewlett-Packard). The transducer was positioned on the left anterior side of the chest. At first, longitudinal images of the heart were obtained, including the left ventricle, atrium, the mitral valve and the aorta, followed by the cross-sectional images from the plane of the base to the left ventricular apical region. M-mode tracings were obtained at the level below the tip of the mitral valve leaflets at the level of the papillary muscles. All of two-dimensional images, M-mode tracings and Doppler curves were recorded on videotape for later analysis. We calculated the fractional shorting (FS) as a measure of systolic function, according to the M-mode tracing from the cross-sectional view: maximal LV end-diastolic diameter (at the time of maximal cavity dimension), minimal LV end-systolic diameter (at the time of maximum anterior motion of the posterior wall), FS (%) = {(LVEDD-LVESD) / LVEDD} x 100.
All measurements were averaged for 3 consecutive cardiac cycles.

Preliminary results

Experiments established the feasibility of growing mouse ESC, as well differentiating ESC within embryoid bodies and neonatal cardiomyocytes into 3D-fibrin gel systems. Figure 1 shows a pictoral representation of the different multi-well (Fig. 1a) systems used to produce the 3D-fibrin gels.
Furthermore, the ongoing studies on the differentiation of EPC show the importance of the presence of vascular endothelial growth factor (VEGF) in the differentiation of the stem cells into mature endothelial cells. Indeed, EPC have the capability to create tube-like constructs in fibrin gels in presence of the epidermal growth factor (EGF).

The feasibility of fixing empty 3D fibrin gel systems on the left ventricle of normal hearts. Furthermore, in an attempt to engraft a 3D fibrin gel system containing undifferentiated ESC, cell survival and proliferation for up to 2 weeks were observed.

Cell therapy of heart failure is presently performed with adult stem cells (form bone marrow or skeletal muscle compartment) has entered recently clinical phase 1 trials. However, cell injection via a syringe is highly ineffective and results in the loss of more than 95% of the cells. The cardiac patches allow a better survival and the correct insertion of the appropriate cells where the tissue need to be regenerated. Furthermore, the vascularization of these tissues is of main importance for the repopulation of the damaged heart and thus the patient survival. Another crucial advantage is the fact that the gels are formed by extracellular matrix proteins present in normal tissues and biodegradable by endogenous and cell-released proteases.

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